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Molecular cloning and characterization of a novel truncated form (ClC-2β) of ClC-2α (ClC-2G) in rabbit heart

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Abstract Two cDNAs encoding rabbit heart Cl^- channels (rabClC-2β and rabClC-2α) were isolated by a PCR cloning strategy. RabClC-2β is a novel cDNA consisting of 2998 bp and encoding the 822-amino acid protein, while rabClC-2α is identical to previously reported ClC-2G. RabClC-2β is 68 amino acids truncated from NH_2-terminus of rabClC-2α, but all 13 putative hydrophobic domains are conserved in rabClC-2β. Although rabClC-2α was suggested to be activated by extracellular hypotonicity, expression of rabClC-2β in *Xenopus* oocytes induced large Cl^- currents even in the absence of extracellular hypotonicity. Induction of external hypotonicity did not further increase the amplitude of membrane currents. On the other hand, as similar to rabClC-2α, rabClC-2β current was augmented by PKA activation. Thus, different RNA processing of the same gene appears to provide two highly homologous PKA-activated Cl^- channels with or without responsiveness to cell swelling in rabbit heart.

Key words: Chloride channels; Heart; Clone; Alternative splicing; Swelling

1. Introduction

A variety of potentially important Cl^- currents have been described in the heart, and they are suggested to play pathophysiological roles by modulating the excitability of cardiac myocyte [1–9]. Membrane depolarization induced by a PKA-activated Cl^- current may result in development of severe arrhythmias associated with acute myocardial infarction [1–3]. A swelling-induced Cl^- current contributes to the pacemaker potential in sino-atrial nodal cells, and activation of this current results in acceleration of cardiac heart rate [7,8]. Although it is clear that Cl^- currents contribute to cardiac electrical activity, data on the molecular aspects of cardiac Cl^- channels are limited [10–12]. In rats and humans, various Cl^- channels are encoded by a family of genes, a ClC family [13–19]. Because cardiac Cl^- currents have been best characterized in rabbits, we made attempts to isolate Cl^- channel cDNAs belonging to ClC family from rabbit hearts. We report here the successful isolation of two highly homologous Cl^- channel cDNAs from rabbit hearts. They appear to be products of different RNA processing of the same gene and to encode PKA-activated Cl^- channels with different responsiveness to cell swelling.

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Abbreviations: Cl^-, chloride; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; GTC, guanidium thiocyanate; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecylsulfate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; 9-AC, 9-anthracene carboxylic acid; *I–V*, current-to-voltage.

2. Materials and methods

2.1. RT-PCR

PCR primers used were: *sense strand,* 5′-CCGAATTCGG(G/C)TC(T/C)GG(A/C)(A/C)TCCCNGA(G/A)(A/C)TGAA(G/A)A C; *antisense strand,* 5′-CCGGATCCNACCTC(T/A/G)ATGCTGAAN AG(G/C)ACNCC. One mg of total RNA extracted from rabbit hearts with the GTC–phenol–chloroform methods [20] was reversed-transcribed with Superscript II reverse transcriptase (Gibco BRL). The synthesized cDNA was used for subsequent PCR in the following profile: 94°C for 45 s, 50°C for 45 s, 72°C for 1.5 min, 40 cycles. The PCR product was cut with *Eco*RI and *Bam*HI on both ends, ligated into *Eco*RI- and *Bam*HI-cut pSPORT 1 (Gibco BRL), and then sequenced.

2.2. Library construction and sequencing

An oligo(dT)-primed directional rabbit atrial cDNA library in λgt22A (Superscript cDNA synthesis kit, Gibco BRL) was prepared and was screened under high stringency (wash with a final stringency of 0.1 × SSPE, 0.1% SDS at 68°C) with PCR products labeled with [α-^{32}P]dCTP. Positive clones isolated were cut with *Not*I and *Sal*I, and inserts were subcloned into *Not*I- and *Sal*I-cut pSPORT 1. Nested deletion clones for a cDNA with the longest insert (cDNA2-3-1) were prepared for both sense- and antisense-strands using the Erase-A-Base system (Promega) and sequenced.

2.3. Northern analysis

Preparation and analyses of mRNA were performed essentially as described [21]. Ten mg of poly(A)$^+$ RNA extracted from rabbit atrium and ventricle were electrophoresed in agarose gel containing formaldehyde. After transfer to nylon membranes, blots were hybridized with a cDNA2-3-1 labeled with [α-^{32}P]dCTP with the random primer labeling system (Takara) and were washed with a final stringency of 0.1 × SSPE, 0.1% SDS at 68°C.

2.4. Primer extension

An antisense oligonucleotide primer was labeled at the 5′-OH end with [γ-^{32}P]ATP using T4 polynucleotide kinase. Poly(A)$^+$ RNA (2.5 mg) were incubated overnight at 53°C with 5×10^5 cpm of a primer in hybridization buffer (10 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA). The sample was precipitated and extended with Superscript II reverse transcriptase (10 units; Gibco BRL) for 60 min at 42°C in a solution containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM

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$MgCl_2$, and 0.5 mM each dNTP. The extended fragments were analyzed by urea/5% polyacrylamide gels.

2.5. RT-PCR to examine tissue distribution

A 0.5 μg of poly(A)$^+$ RNA isolated from various tissues of rabbits were reverse-transcribed with Superscript II reverse transcriptase. One tenth (v/v) of the synthesized cDNA was used for subsequent PCR in the following profile: 94°C for 45 s, 61°C for 45 s, 72°C for 1.5 min, for various cycles. Quantification of PCR products were done by incorporation of [α-^{32}P]dCTP. After PCR products were electrophoresed in agarose gels, bands with corresponding size were cut out, and the count of incorporated radioisotope was measured by liquid scintillation analyzer (Packard, 2000CA).

2.6. Recording of Cl^- current expressed in Xenopus *oocytes*

After linearization of a cDNA2-3-1 subcloned into *Not*I- and *Sal*I-cut pSPORT 1 with *Not*I, capped RNA was synthesized in vitro using T7 RNA polymerase. 10–50 ng of transcript was injected into *Xenopus* oocytes prepared as described previously [22]. Injected oocytes were incubated for 2–4 days at 20°C in modified Barth's solution. Membrane currents were recorded by two electrode voltage clamp using a Dagan 8500 amplifier (Dagan Corp.) at a room temperature of 24–26°C in ND96 solution containing (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and HEPES 5. Extracellular hypotonicity was achieved by diluting ND96 with equal volume of distilled water (1/2 ND96). Data were collected and analyzed using pClamp software (Axon Instruments Inc.).

3. Results

PCR products of expected size were subcloned and sequenced (see Fig. 2A for primer sites). Sequencing revealed the existence of PCR clone in rabbit atrium that was highly homologous but not completely identical to that of rClC-2 (93% nucleotide identity). Using this PCR clone as a probe, we isolated 4 positive clones, and fully sequenced a clone containing the longest insert (cDNA2-3-1), which consists of 2998 bp and encodes the 822-amino acid protein of a relative molecular mass of 90,108 Da (Fig. 1). The Kyte-Doolittle hydrophobicity profile [23] of this clone was also very similar to those of other ClC families, containing 13 conserved hydrophobic domains (D1 through D13) (Fig. 2A). The overall amino acid identity of this clone was about 97% with rabbit ClC-2G [24], 81% with rClC-2, 57% with *Torpedo* ClC-0, 57% with rat ClC-1, 47% with rat ClC-3, 57% with rat ClC-K1, and 55% with rat ClC-K2L (Fig. 2A). There are three potential N-linked glycosylation sites; N_{159}ES, N_{330}RT, and N_{708}FS (Fig. 2A) [25]. Several putative consensus sequences for protein kinases exist; serine at position 575 ($RRQS_{575}KQKRR$) is a strong consensus sequence for phosphorylation by both PKA and PKC; $RGCS_{626}$, RKS_{673}, RTS_{726}, RDS_{784}, and $REGS_{813}$ are also potential phosphorylation sites by PKA (Fig. 2A) [26]. The difference between cDNA2-3-1 and ClC-2G was localized to their extreme 5′-ends; nucleotide identity of 35 bp from the 5′-end of cDNA2-3-1 and 128 bp of rabbit ClC-2G was decreased to 20% (Fig. 2B). Because of this difference, initiation methionine for cDNA2-3-1 appears to be assigned to methionine$_{69}$ of ClC-2G, and a putative protein encoded by cDNA2-3-1 was 68 amino acids truncated from that encoded by ClC-2G. However, all 13 putative hydrophobic domains in rabbit ClC-2G are conserved in cDNA2-3-1.

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                                        -143 CCACGCGTCCGCGGACGCGTGGG -121
CGGACGCGTGGGGCCGCTCGCATTCGCCTGGGAGGGCCTGAGCCCTGGAGGAGCCCGCCC  -61
TCCCCTCGGACTCCCCCAGAGCTCCTGGAATATGGACAGAGCCGCTGTGCCCGATGCCGC   -1
ATGTGCTCTGTGCGCTGCCACAAGTTCCTGGTGTCCAGGGTTGGTGAAGACTGGATCTTC   60
MetCysSerValArgCysHisLysPheLeuValSerArgValGlyGluAspTrpIlePhe
CTGGTCCTGCTGGGGCTCCTCATGGCGCTGGTCAGCTGGGCCATGGATTACGCCATCGCT  120
LeuValLeuLeuGlyLeuLeuMetAlaLeuValSerTrpAlaMetAspTyrAlaIleAla
GCCTGTCTGCAGGCTCAGCAGTGGATGTCCCGGGGCCTGAACACCAACCTCCTGCTCCAG  180
AlaCysLeuGlnAlaGlnGlnTrpMetSerArgGlyLeuAsnThrAsnLeuLeuLeuGln
TACCTGGCCTGGGTCACCTACCCCGTCGTCCTCATCACTTTCTCCGCCGGATTCACACAG  240
TyrLeuAlaTrpValThrTyrProValValLeuIleThrPheSerAlaGlyPheThrGln
ATCCTGGCCCCTCAGGCTGTGGGGTCCGGCATCCCCGAGATGAAAACCATCTTACGGGGA  300
IleLeuAlaProGlnAlaValGlySerGlyIleProGluMetLysThrIleLeuArgGly
GTGGTGCTGAAAGAATACCTCACCCTCAAGACCTTCGTAGCCAAGGTCATCGGGCTGACC  360
ValValLeuLysGluTyrLeuThrLeuLysThrPheValAlaLysValIleGlyLeuThr
TGTGCCTTGGGCAGTGGGATGCCACTGGGCAAAGAGGGCCCTTTTGTGCATATTGCCAGC  420
CysAlaLeuGlySerGlyMetProLeuGlyLysGluGlyProPheValHisIleAlaSer
ATGTGCGCCGCCCTTCTCAGCAAGTTCCTCTCCCTCTTTGGGGGCATCTATGAGAACGAG  480
MetCysAlaAlaLeuLeuSerLysPheLeuSerLeuPheGlyGlyIleTyrGluAsnGlu
TCTCGGAACACAGAGATGCTGGCTGCCGCCTGTGCCGTGGGCGTGGGCTGCTGCTTCGCC  540
SerArgAsnThrGluMetLeuAlaAlaAlaCysAlaValGlyValGlyCysCysPheAla
GCCCCCATCGGAGGCGTCCTATTCAGCATTGAGGTCACCTCCACCTTCTTCGCCGTGCGC  600
AlaProIleGlyGlyValLeuPheSerIleGluValThrSerThrPhePheAlaValArg
AACTACTGGCGCGGCTTTTTCGCCGCCACCTTCAGCGCCTTCATCTTCAGGGTCTTGGCT  660
AsnTyrTrpArgGlyPhePheAlaAlaThrPheSerAlaPheIlePheArgValLeuAla
GTCTGGAACCGGGATGAAGAGACCATCACCGCGCTCTTCAAAACCCGATTCCGGCTCGAC  720
ValTrpAsnArgAspGluGluThrIleThrAlaLeuPheLysThrArgPheArgLeuAsp
TTCCCCTTCGACCTGCAGGAGCTGCCAGCCTTTGCGGTGATTGGAATCGCTAGTGGCTTC  780
PheProPheAspLeuGlnGluLeuProAlaPheAlaValIleGlyIleAlaSerGlyPhe
GGAGGAGCCCTCTTTGTCTACCTGAACCGGAAGATCGTGCAGGTGATGAGGAAGCAGAAG  840
GlyGlyAlaLeuPheValTyrLeuAsnArgLysIleValGlnValMetArgLysGlnLys
ACCATCAATCGCTTCCTGATGCGAAAACGCCTGCTCTTCCCAGCCCTGGTCACCCTGCTC  900
ThrIleAsnArgPheLeuMetArgLysArgLeuLeuPheProAlaLeuValThrLeuLeu
ATCTCCACTCTGACCTTCCCTCCTGGCTTTGGACAGTTCATGGCTGGACAGCTCTCACAG  960
IleSerThrLeuThrPheProProGlyPheGlyGlnPheMetAlaGlyGlnLeuSerGln
AAGGAAACACTGGTCACCCTGTTTGACAACCGGACCTGGGTCCGCCAGGGCCTGGTGGAG 1020
LysGluThrLeuValThrLeuPheAspAsnArgThrTrpValArgGlnGlyLeuValGlu
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GluLeuGluProProSerThrSerGlnAlaTrpSerProProArgAlaAsnValPheLeu
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ThrLeuValIlePheIleLeuMetLysPheTrpMetSerAlaLeuAlaThrThrIlePro
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ValProCysGlyAlaPheMetProValPheValIleGlyAlaAlaPheGlyArgLeuVal
GGAGAGAGCATGGCTGCCTGGTTCCCCGACGGGATTCACACAGACAGCAGTACTTACAGG 1260
GlyGluSerMetAlaAlaTrpPheProAspGlyIleHisThrAspSerSerThrTyrArg
ATCGTGCCCGGGGGCTATGCTGTGGTGGGGGCGGCTGCTCTGGCAGGAGCGGTGACCCAC 1320
IleValProGlyGlyTyrAlaValValGlyAlaAlaAlaLeuAlaGlyAlaValThrHis
ACAGTGTCCACGGCCGTGATCGTGTTCGAGCTCACGGGCCAGATCGCCCATATCCTGCCC 1380
ValMetIleAlaValIleLeuAlaAsnAlaValAlaGlnSerLeuGlnProSerLeuTyr
GTCATGATCGCTGTCATCCTGGCTAACGCCGTTGCGCAGAGCCTGCAGCCCTCCCTCTAC 1440
ThrValSerThrAlaValIleValPheGluLeuThrGlyGlnIleAlaHisIleLeuPro
GACAGCATCATCCGAATCAÃGAAGCTCCCCTATCTGCCTGAGCTGGGCTGGGGCCGCCAC 1500
AspSerIleIleArgIleLysLysLeuProTyrLeuProGluLeuGlyTrpGlyArgHis
CAGCAGTACCGCGTGCGAGTGGAAGACATCATGGTGCGGGATGTTCCCCACGTGGCGCTC 1560
GlnGlnTyrArgValArgValGluAspIleMetValArgAspValProHisValAlaLeu
AGCTGCACCTTCCGGGACCTGCGGCTGGCACTGCACAGGACCAAGGGCCGCACGCTGGCC 1620
SerCysThrPheArgAspLeuArgLeuAlaLeuHisArgThrLysGlyArgThrLeuAla
CTGGTGGAGTCTCCTGAGTCTATGATCCTTCTGGGTTCCATCGAGCGCACGCAGGTGGTG 1680
LeuValGluSerProGluSerMetIleLeuLeuGlySerIleGluArgThrGlnValVal
GCGCTGCTGGCTGCCCAGCTGAGCCCGGCCCGCCGGCGGCAGTCCAAGCAGAAGCGCAGG 1740
AlaLeuLeuAlaAlaGlnLeuSerProAlaArgArgArgGlnSerLysGlnLysArgArg
GTGGCCCACACCTCTCCACCGTCCTGTCAGGAGAGTCCCCCCAGCCCTGAGACTTCCGTC 1800
ValAlaHisThrSerProProSerCysGlnGluSerProProSerProGluThrSerVal
TGCTTCCAGGTGAAGGCCGAGGACGCCCAGGGGGAGCCTCACAAGCCCCTAAAGCCTGCT 1860
CysPheGlnValLysAlaGluAspAlaGlnGlyGluProHisLysProLeuLysProAla
CTCAAAAGGGGGTGCAGCAACTCCGTGAACCTCGGGGAGAGTCCCACAGGGCACGTGGAG 1920
LeuLysArgGlyCysSerAsnSerValAsnLeuGlyGluSerProThrGlyHisValGlu
TCGGCCGGCATCGCGCTCAGGAGCCTCTTCTGTGGCAGTCCCCCTCCAGAGGCCGCGTCA 1980
SerAlaGlyIleAlaLeuArgSerLeuPheCysGlySerProProProGluAlaAlaSer
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GluSerGluLysSerGluSerSerGluLysArgLysSerLysArgValArgIleSerLeu
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GlnGlnLeuAspGluProValAsnPheSerAspCysLysIleAspProAlaProPheGln
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LeuValGluArgThrSerLeuHisLysThrHisThrIlePheSerLeuGluGlyValAsp
CACGCATACGTCACCAGCATCGGCAGGCTCATTGGAATCGTTACGCTAAAGGAGCTCCGG 2260
HisAlaTyrValThrSerIleGlyArgLeuIleGlyIleValThrLeuLysGluLeuArg
AAGGCCATTGAGGGCTCTGTCACAGCACAGGGTGTGAAGGTCCGGCCGCCCCCTCGCCAGC 2320
LysAlaIleGluGlySerValThrAlaGlnGlyValLysValArgProProLeuAlaSer
TTCCGAGACAGTGCTCACCAGCAGCAGTGACACAGAGACCACCGAGGTGCACGCGCTTTGG 2380
PheArgAspSerAlaThrSerSerSerAspThrGluThrThrGluValHisAlaLeuTrp
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GlyProArgSerArgHisGlyLeuProArgGluGlySerProSerAspSerAspAspLys
TGCCAATGAGCCCCTGCGTGGGCTGTGGCTGTCAGGCCAGATTATATAGCTCTCCCATGC 2500
CysGln
CGCCTCCTGCCCTGGGAAAGCAGAAGACAGCCGCAGCCCCAGCCCCACCTCCAGACCCGG 2560
CCTGCCAACGTCTCCCAGAGCTCATCCTGCCTGGAAACTGACCCAGCACCTCCTGCAGCA 2620
GGTGTCCCAAGGGCAAGATCAGCACTGCCCTGGTAGCATGGGGGTGGGGTCACTTGACCC 2680
CCCTACCCCGTTGAGGGAAAGGGATAGAACTAAGATGGGTTTATACTGGAACCTCCAATG 2740
ACCAGATGTATATAGAGATTTACAAAGATTTTTATATTAATTTAATAAAACAAATTCTTA 2800
AATAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                           2835
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of rabClC-2β.

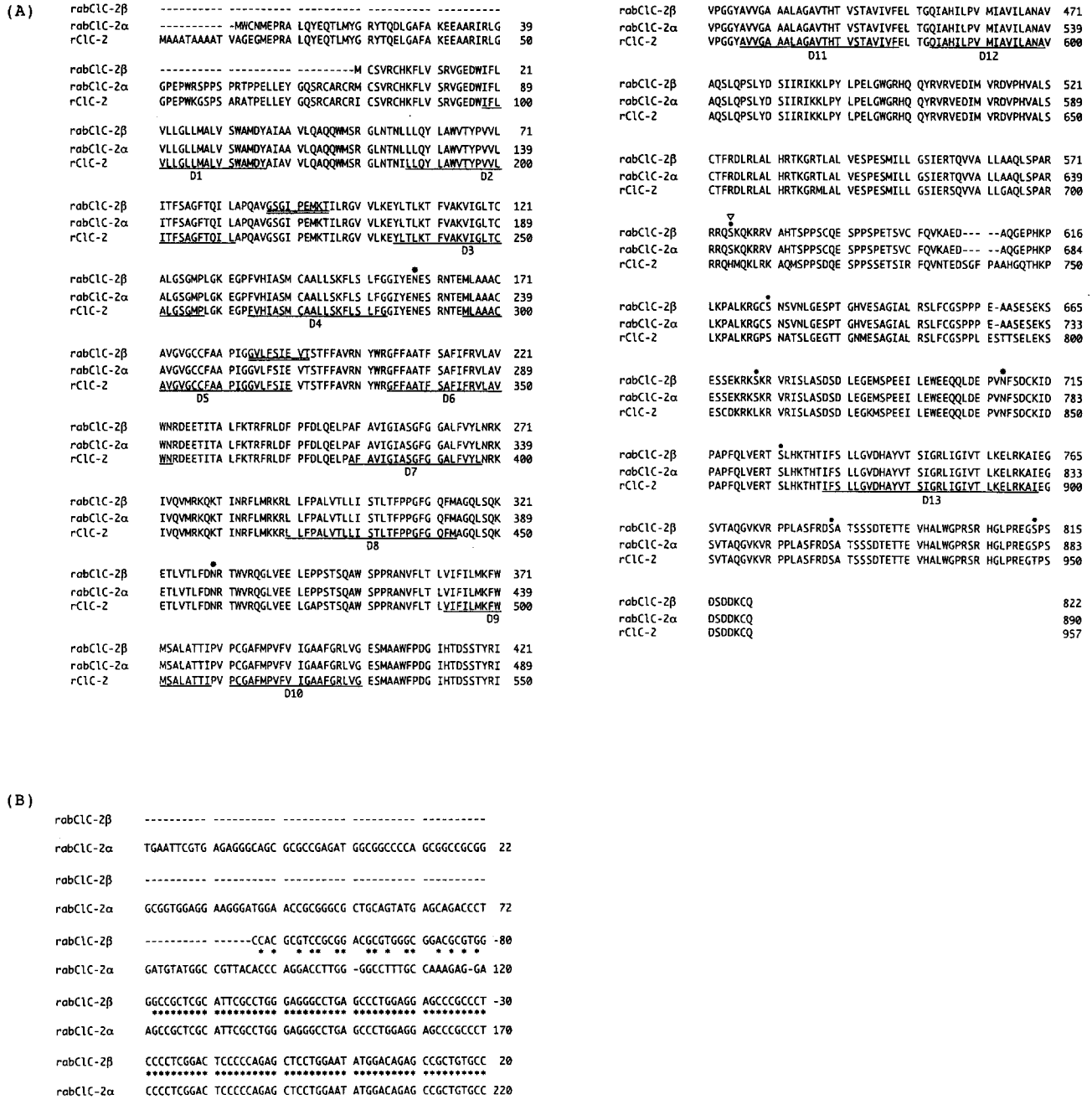

Fig. 2. (A) Amino acid sequence of rabClC-2β and its alignment with that of rabClC-2α (ClC-2G) and rClC-2. Putative transmembrane spanning domains are underlined, potential N-linked glycosylation sites are indicated by asterisks, consensus PKA and PKC phosphorylation sequences by closed circles and open inverted triangles, respectively, and PCR primers by double-underlining. (B) Alignment of nucleotide sequence of the 5′-end of rabClC-2β and -2α. Identical nucleotides between the two cDNAs are indicated by asterisks.

Northern analysis revealed a positive band at ~3.1 kb (Fig. 3A). Because cDNA2-3-1 had short NH_2-terminus coding sequence preceding a putative 1st hydrophobic domain (D1) compared to rClC-2 and ClC-2G, primer extension was further employed to search for possible 5′-terminal coding and 5′-untranslated sequence. A primer was made at 211b and 324b from the 5′-end of cDNA2-3-1 and ClC-2G, respectively. Three positive bands at around 110b, 220b, and 360b were noticed (Fig. 3B). The length of the latter two bands were close to the length between 5′-ends of cDNA2-3-1 and ClC-2G and the site of primer, respectively. Thus, double-strand DNAs corresponding to these two bands were made, subcloned into *Sma*I-cut pSPORT 1, and were sequenced. Fragment DNA eluted from the band at ~220b corresponds to the 5′-end of cDNA2-3-1, and that eluted from the band at ~360b corresponds to the 5′-end of ClC-2G. These findings were confirmed by the rapid amplification of cDNA end (RACE) using the 5′ RACE system (Gibco BRL). Neither primer extension nor 5′-RACE extended beyond the 5′-end of cDNA2-3-1. Taken together with the finding that the size of extended fragment by primer extension was

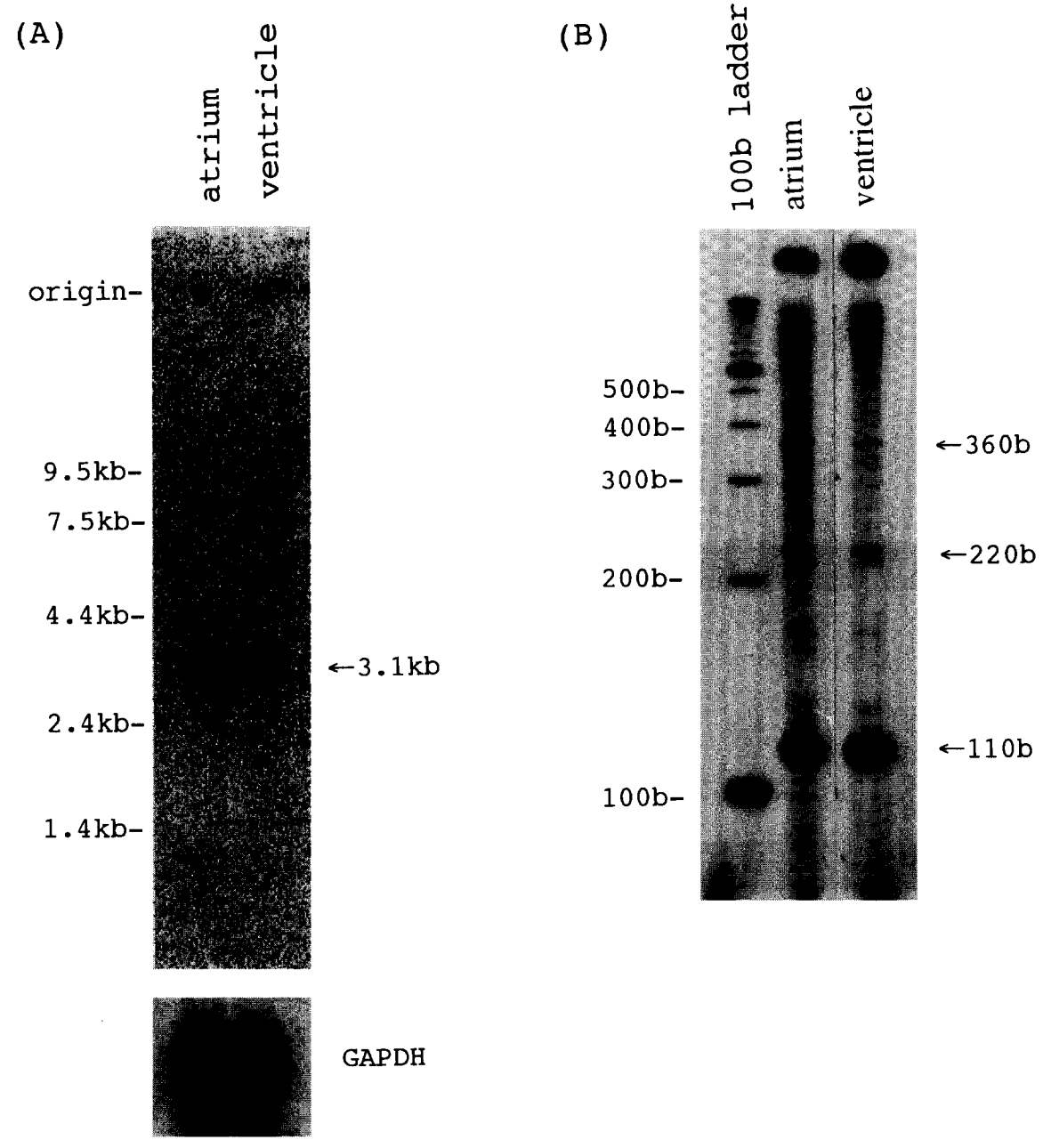

Fig. 3. (A) Northern blot analysis of rabClC-2β in rabbit atrium and ventricle. 10 mg of poly(A)$^+$ RNA were run on each lane. Equal loading was checked using a probe for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Result of primer extension.

similar to the size from the 5′-end of cDNA2-3-1 and the site of primer, we suggest that the cDNA2-3-1 shown in Fig. 1 may cover almost full-length of this mRNA. In order to confirm the expression of ClC-2G in the heart, a pair of PCR primers whose expected products covered the entire coding region were made; *sense primer* was 5′-GAATTCGTGAGAGGGCAGCG, and *antisense primer* was 5′-GGATCCGGGGTAGCGGGGTCA-AGTG. PCR products were cut on both ends with *EcoR*I and *BamH*I, subcloned into *EcoR*I- and *BamH*I-cut pSPORT 1, and were sequenced on both strands. The PCR products had complete nucleotide identity with ClC-2G. Since ClC-2G is not localized to stomach, we propose to designate this cDNA as rabClC-2α, and cDNA2-3-1 as rabClC-2β. Based on the complete nucleotide identity between rabClC-2α and rabClC-2β except a short region of extreme 5′-end, it appears virtually certain that both mRNAs are derived from the same gene and are products of different RNA processing.

In order to differentially characterize tissue distribution of rabClC-2α and rabClC-2β, RT-PCR analysis was performed using either of two sets of primers (Fig. 4A). As an internal control, a set of primers that can amplify a part of rabbit β-actin (455 bp) were included in a PCR mixture. Fig. 4B shows ethidium bromide stained agarose gels loaded with PCR products, and Fig. 4C summarizes incorporation of [α-^{32}P]dCTP into a part of rabClC-2α or -2β relative to a part of rabbit β-actin. Since we confirmed that PCR products were exponentially am-

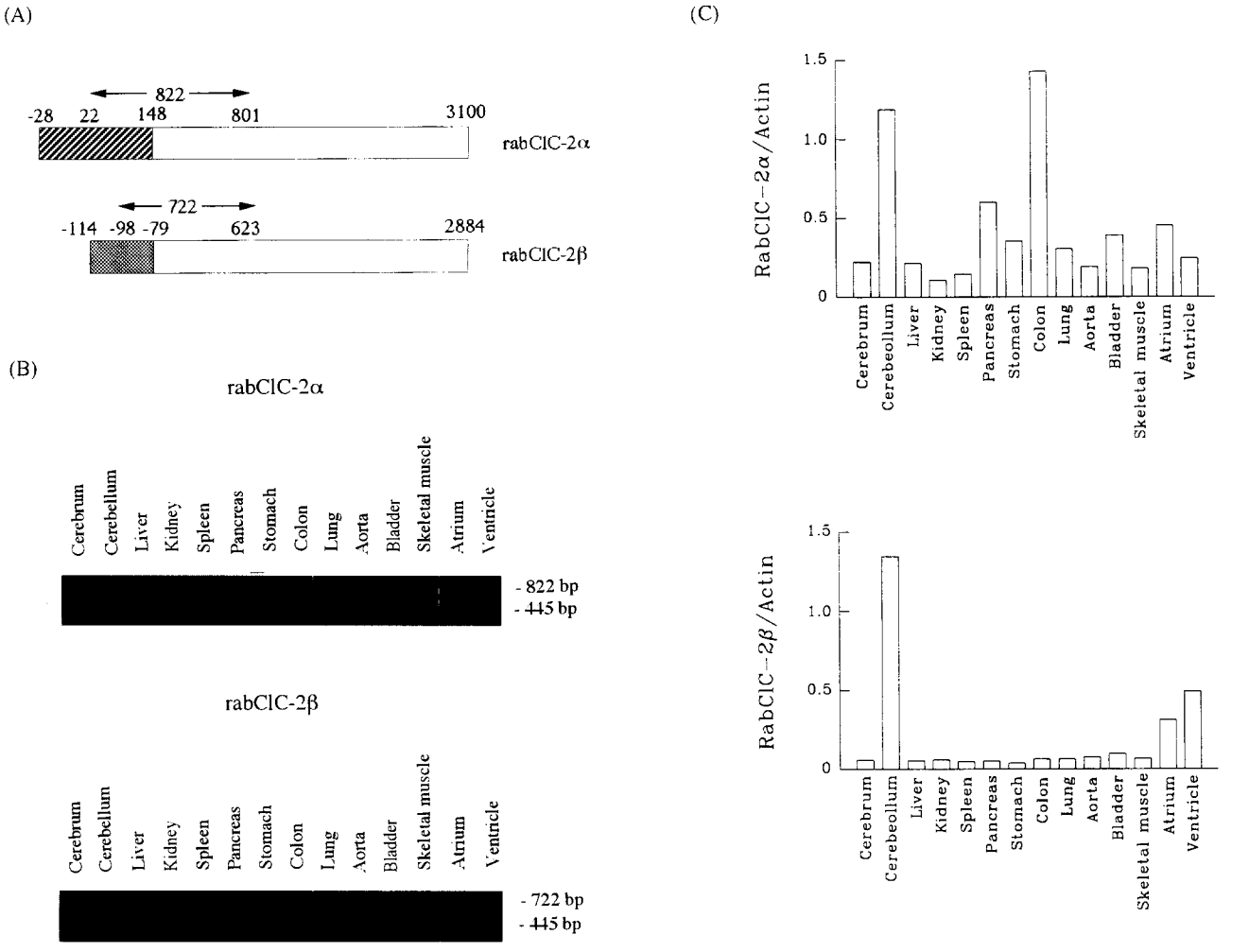

Fig. 4. (A) Designs of a set of primers to differentially examine the abundance of message for rabClC-2α and -2β. (B) Tissue distribution of rabClC-2α and -2β determined by RT-PCR. Ethidium bromide stained agarose gels in which PCR products were loaded. Bands of 822 bp correspond to an amplified part of rabClC-2α, 722 bp bands to a part of rabClC-2β, and 455-bp bands to a part of rabbit β-actin as an internal control. (C) Quantification of products of RT-PCR expressed as an incorporated radioisotope into rabClC-2α or -2β relative to an incorporation into rabbit β-actin.

plified by PCR cycle numbers from 20 to 35 (data not shown), only data using 30 PCR cycles are presented. Similar density of ethidium bromide stained 455-bp bands in each lane suggested inclusion of equal amount of poly(A)$^+$ RNA, their integrity, and equal efficiency of reverse transcription. RabClC-2α and -2β show distinct pattern of tissue distributions. Although rabClC-2α was predominantly expressed in cerebellum and colon, it was also expressed with an abundance of 1/10 to 1/2 of those in cerebellum and colon in every tissue. On the other hands, a message for rabClC-2β was observed predominantly in cerebellum and an abundance of that in ventricle and atrium were ~30% of that in cerebellum. Other tissues shows very little abundance of a rabClC-2β message; less than 5% of that in cerebellum.

Xenopus oocytes injected with a cRNA of rabClC-2β exhibited large membrane currents, while those injected with water exhibited minimal membrane currents (Fig. 5A and B). A reversal potential of expressed currents (−25 mV) was near the equilibrium potential of Cl ion in *Xenopus* oocytes, and currents were inhibited by externally applied 1 mM 9-AC (Fig. 5C) and 1 mM DIDS (data not shown) suggesting expression of Cl^- currents. Shifts in reversal potential from −25 mV to −15 mV when external Cl^- was decreased from 103.6 mM to 55.6 mM (see Fig. 5E and F) further suggest that Cl^- is a charge carrier of this current. Membrane currents were enhanced by application of 10 mM forskolin in the bath solution (Figs. 5D and F). Extracellular hypotonicity did not change the amplitude of membrane currents, but shifted the reversal potential to a positive potential due to the change in $[Cl^-]_o$ from 103.6 mM to 55.6 mM (Figs. 5E and F).

4. Discussion

We isolated two highly homologous members of ClC Cl^- channel family from rabbit atrium. RabClC-2β is a novel cDNA encoding the 822-amino acid protein, while rabClC-2α is identical to previously reported ClC-2G [24]. They have 97% overall amino acid identity, and rabClC-2β is 68 amino acids truncated from NH_2-terminus of rabClC-2α. Thus, they appear to be products of different RNA processing of the same gene,

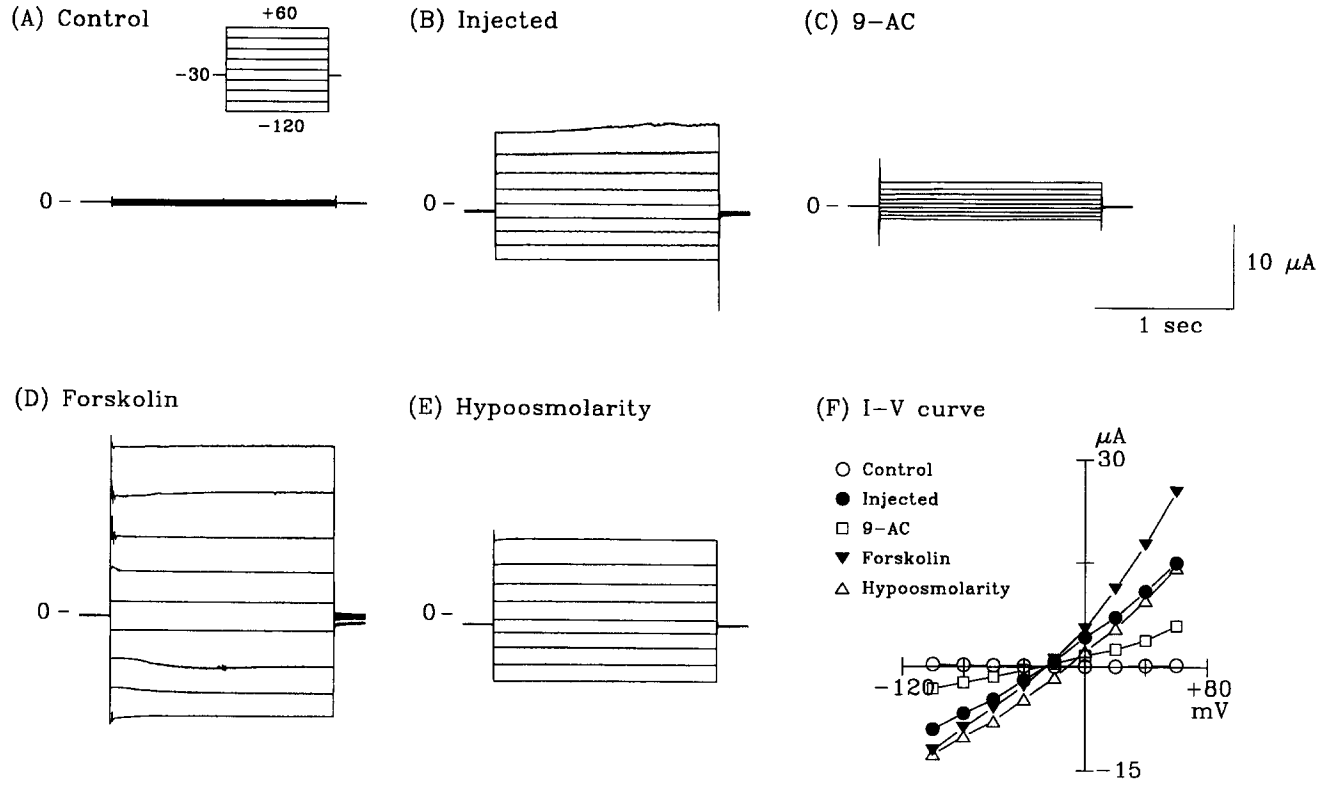

Fig. 5. (A and B) Membrane currents recorded in *Xenopus* oocytes previously injected with water (A) or rabClC-2β cRNA (B). (Inset) Voltage clamp protocol. (C, D and E) Membrane currents recorded oocytes previously injected with rabClC-2β in the presence of 1 mM 9-AC (C), 10 mM forskolin (D), or in extracellular hypotonic solution (1/2 ND96) (E). (F) The *I–V* relationships.

and they show different tissue distribution patterns. However, without sequence analysis and knowledge of intron/exon organization of the gene, it is not known whether rabClC-2α and -2β are alternative splicing products of the common initial transcripts or whether they are products derived from distinct alternative promoter regions.

Expressed rClC-2 current was enhanced when oocytes were swollen by superfusion with hypotonic solution [27]. Deletion mutation from 5′-end of rClC-2 loses sensitivity to cell swelling, resulting in an open channel even in the absence of cell swelling [27]. Thus, 5′-region of rClC-2 was suggested as a critical site for determination of sensitivity to cell swelling [25]. 5′-Region of rabClC-2α showed very high amino acid identity to rClC-2, suggesting a responsiveness of rabClC-2α to extracellular hypotonicity. On the other hand, a short region of NH_2-terminus is truncated in rabClC-2β. Oocytes injected with rabClC-2β cRNA showed large currents in ND96, and hypotonic stimulation did not enhance membrane currents. On the other hand, rabClC-2α and -2β are similar in terms of their responsiveness to PKA. Putative consensus sequences for PKA phosphorylation gather to the cytosolic COOH-terminus of the two cDNAs, and they are conserved between the two cDNAs. Expressed rabClC-2α current and -2β current were both augmented by PKA activation. Thus, in rabbit heart and probably in cerebellum, the alternative RNA processing of the same Cl^- channel gene provides two highly homologous PKA-activated Cl^- channels with or without responsiveness to cell swelling. The alternative RNA processing, therefore, may provide a molecular basis for functional diversity of Cl^- channel in rabbit heart.

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